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14. ABSTRACT

The epidermis of the mouse lacking the vitamin D receptor (VDR) is susceptible to chemical and UVB induced tumor formation. In previous studies we determined that the hedgehog (HH) and wnt/β-catenin pathways were activated in the skin of VDR null mice. These pathways when activated promote proliferation and inhibit differentiation, suggesting the hypothesis that they underlie the predisposition of VDR null mouse epidermis to tumor formation following chemical or UVB induced mutations. Accordingly we developed mice in which the wnt/β-catenin pathway was either constitutively activated by deletion of exon 3 of the β-catenin gene or inactivated by deletion of exons 2-6 of the β-catenin gene and mice in which the HH pathway was inactivated by deletion of the Shh gene. These were then bred with mice with the floxed VDR. At four weeks these deletions were achieved using a keratinocyte specific and tamoxifen regulated (ERT2 K14 driven cre) cre recombinase. The phenotypes were remarkable with disruption of hair follicles and hair follicle cycling. Survival for a number of these genotypes was limited, precluding completing the 40wk UVB time course for all but the VDR/β-catenin knockout. This double knockout also developed tumors indicating that deletion of β-catenin did not show the expected protection. We have also evaluated the acute response of these mouse models to UVB, but the results are pending.

15. SUBJECT TERMS
epidermal tumors, keratinocytes, vitamin D receptor, sonic hedgehog, β-catenin, UVB

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Introduction

This project was designed to test the hypothesis that the vitamin D receptor (VDR) functions as a tumor suppressor with respect to epidermal tumor formation by blocking the β-catenin and hedgehog pathways, key pathways in keratinocyte proliferation that if left unchecked lead to tumor formation. We proposed two main objectives:

1. Determine whether mice lacking VDR but with constitutively active β-catenin show accelerated tumor formation following UVB treatment, whereas those with an inactivated β-catenin are protected from tumor formation.
2. Determine whether mice lacking VDR but with constitutively active Hh signaling (ptch null) show accelerated tumor formation following UVB treatment, whereas those with an inactivated Shh are protected from tumor formation.

To achieve the first objective we developed mice in which the VDR gene and the β-catenin gene were floxed, the latter in either exons 2-6 to delete the gene or exon 3 to activate the gene product. These mice were then bred with mice expressing a tamoxifen regulated cre recombinase. At 5wks of age tamoxifen was given to remove the floxed portion of the genes. The animals were then subjected to UVB acutely to examine the acute effect of UVB or for 40wks to induce tumor formation. To achieve the second objective we developed mice in which the VDR gene and the sonic hedgehog gene (SHH) were floxed then bred with mice expressing a tamoxifen regulated cre recombinase. As for the first objective tamoxifen was given at 5 wks to remove the floxed portion of the genes, and the animals subjected to UVB acutely to examine the acute effect of UVB. Poor survival precluded the chronic UVB exposure experiments in this objective.

Body

Task 1. **Determine whether mice lacking VDR but with constitutively active β-catenin show accelerated tumor formation following UVB treatment, whereas those with an inactivated β-catenin are protected from tumor formation.** We bred mice homozygous for the floxed VDR with mice homozygous for floxed β-catenin either of exon 3 (which when deleted results in a constitutively active β-catenin) or of exons 2-6 resulting in a β-catenin null when deleted. Deletion of the VDR and the appropriate β-catenin exons was accomplished by breeding the double floxed mice with ER^{T2}-K14-cre recombinase. This cre recombinase is expressed only in basal keratinocytes, and tamoxifen activates it enabling the gene deletions to be temporally controlled. In this study tamoxifen was administered at 4 wks. These mice were then treated acutely or for up to 40wks with UVB using a dose protocol previously reported to induce skin tumors in VDR null mice (but not controls). The breeding produced littermates which have or lack the ER^{T2}-K14-cre recombinase, and the latter served as the "wildtype" controls since they retain the functional genes. The acute (ie. one exposure to 400mJ/cm^2) exposure to UVB was intended to look for differences in cyclobutane pyrimidine dimer (CPD) formation/clearance (markers of DNA damage), hyperplasia, and activation of the β-catenin and Hh pathways, whereas the chronic (3x/wk escalating doses up to 400mJ/cm^2) exposure will look for tumor formation. These tumors were characterized as to type with differentiation markers, proliferation and expression of Hh and β-catenin pathway components.

Results:

1a. *Obtain ACURO approval for the animal studies:* ACORP approval was obtained to perform these experiments

1b. *Develop mice with floxed VDR, exon 3 or exon 2-6 β-catenin, and the combination of floxed genes and breed them with mice expressing the appropriate floxed genes plus ER^{T2}-K14-cre recombinase.* All breeding was successfully accomplished for these experiments

1c. *Treat the litters from the above breeding and those of the single floxed gene controls with 0.2mg tamoxifen/mouse intraperitoneally every other day for 3 injections beginning at 4wks of age.* This has been accomplished for all strains of bioengineered mice. Figure 1 demonstrates the phenotypes of the mice lacking VDR in combination with deletion of β-catenin (*del Ctnnb1)* or constitutive activation of β-catenin (*caCtnnb1)*.

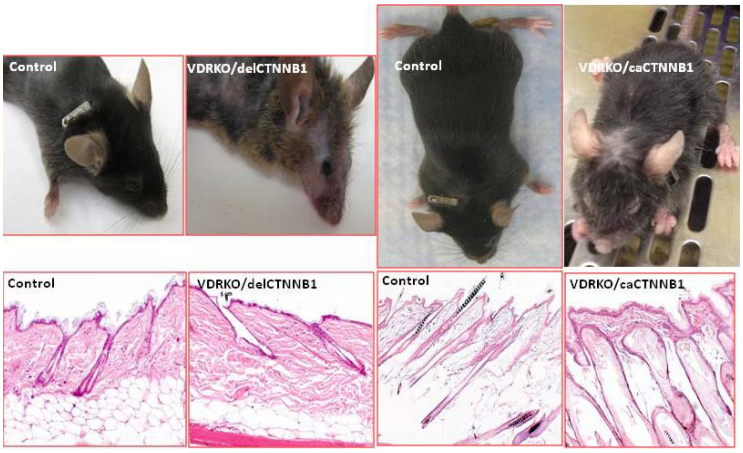


Figure 1. Impact of breeding $^{epid}Vdr^{-/-}$ mice with mice expressing del*Ctnnb1* or ca*Ctnnb1* in their keratinocytes. Tamoxifen was administered at week 4, and these photos and histology were obtained at 8 wks. The controls are littermates lacking cre. The effect of deleting *Vdr* is not yet evident, unlike the impact of manipulating β-catenin levels.

What we found is that the mice lacking β-catenin quickly started losing hair. VDR null mice also developed alopecia but not before several months. So loss of β-catenin accelerated this process. Surprisingly, the mice lacking both VDR and β-catenin (DKO) had better survival than did the mice lacking only β-catenin. Nevertheless, the skin was scaly and hyperproliferative. However, in the DKO, acute UVB failed to further increase proliferation or hyperplasia. In contrast the mice with the ca β-catenin developed a very thick coat of hair at least initially. Histologically this was associated with increased numbers and markedly widened hair follicles (figure 1). However, with time these mice began losing hair, growth slowed, and mortality soared. Because of the mortality rate we have been unable to complete the UVB experiments with the ca β-catenin mice. Therefore we modified the protocol to examine mice which were heterozygous for the ca β-catenin, and survival has improved. These mice are currently under investigation.

1d. *Perform long treatment (40wks) of the mice with UVB starting at 5wks (1 week after tamoxifen injections).* Because of the problems with survival with the ca β-catenin and the β-catenin KO mice we have only been able to carry the DKO mice for the full 40 wk of UVB, although experiments continue with the heterozygous ca β-catenin/VDRKO mice. Surprisingly the DKO mice formed more tumors than the VDRKO mice (figure 2), although the tumors were small and generally benign.

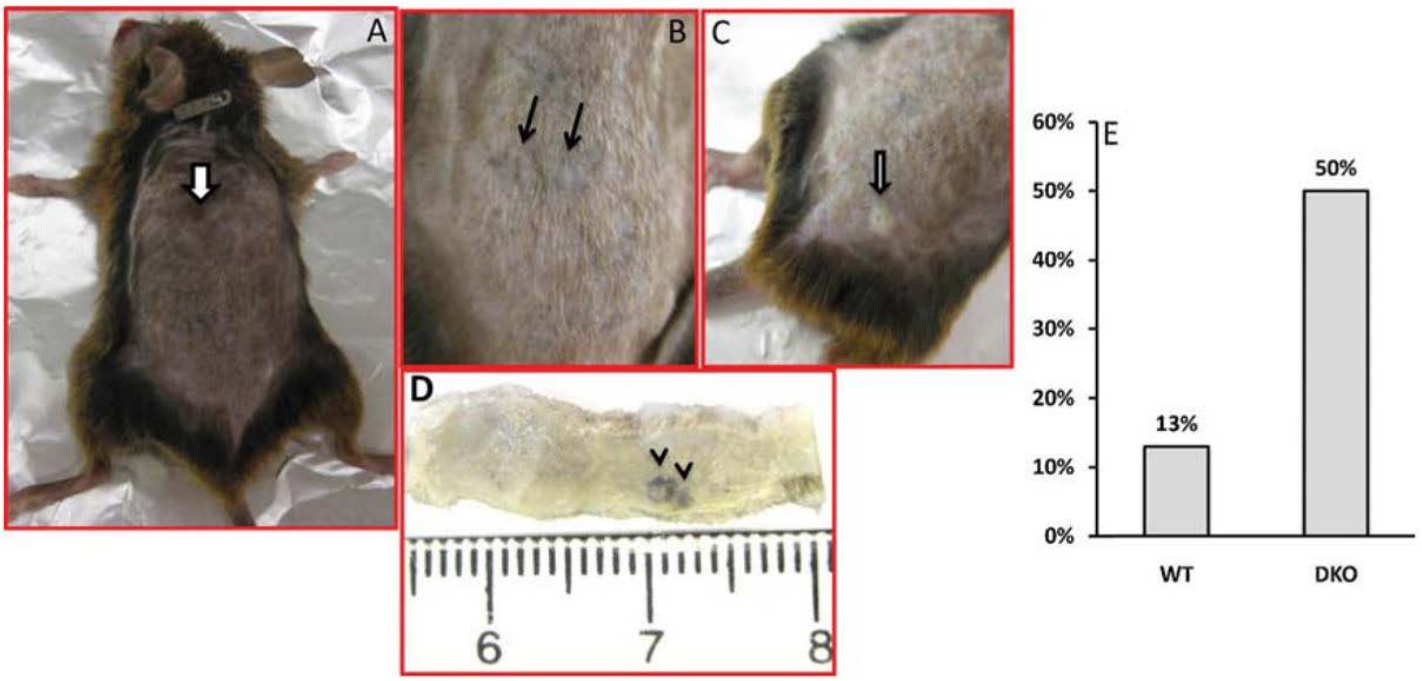
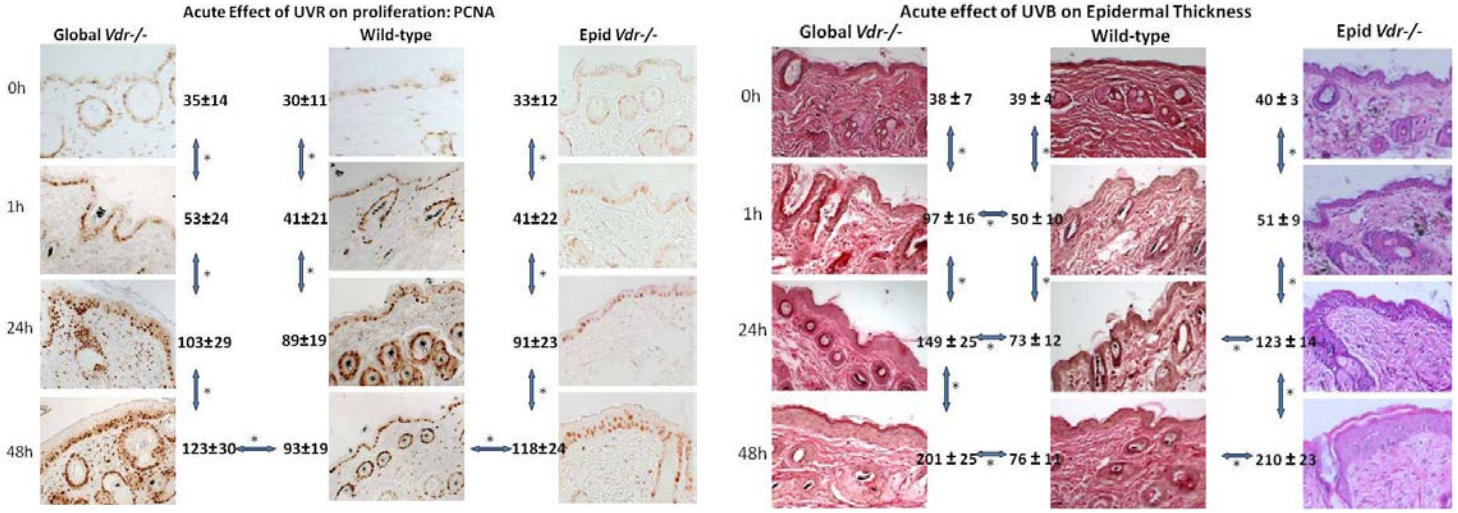


Figure 2. Conditional deletion of VDR and β-catenin in epidermis promotes UVB-induced skin tumors. (A) The whole body of DKO mouse shows pigmentation (arrow) and hair, two solid dark-brown tumors (B and D) and one white, exophytic tumor (C). (E) The graph shows the increased rate (%) of skin tumor formation in DKO vs. WT control.

1e. *Perform acute treatment with a single dose of UVB starting at 5wks (1 week after tamoxifen injections).*Acute UVB exposure was performed in all genotypes that survived the deletion/activation of their genes. However, at the time of writing the histologic analyses are not yet complete. Figures 3 and 4 demonstrate the acute effect of UVB on mice lacking the VDR in their keratinocytes

Figure 3. Acute effects of UVR on epidermal proliferation and hyperplasia in *Vdr$^{-/-}$* mice. Mice lacking *Vdr* globally or specifically in the epidermis were irradiated with 1 dose of 400mJ/cm^2 UVB, and their skin obtained prior to and 1, 24, and 48h after UVR for immunostaining for PCNA, a marker of proliferation, (left panel) or epidermal thickness (right panel). Although the rates of proliferation increased in both wildtype and *Vdr$^{-/-}$* mice through 24h after UVR, by 48h there were significantly greater numbers of proliferating keratinocytes in the *Vdr$^{-/-}$* epidermis. The numbers represent mean +/- SD of 6 images per mouse and 3 mice/group. Similar to that of proliferation, the increase in thickness in the wildtype epidermis increased through 24h, but that in the *Vdr$^{-/-}$* epidermis not only increased faster, but continued to increase throughout the 48h time course reaching a thickness nearly 3 fold that of the control mouse epidermis. Six sections per mouse and 3 mice per genotype were measured at each time point. The numbers represent mean +/- SD of 6 images per mouse and 3 mice/group.



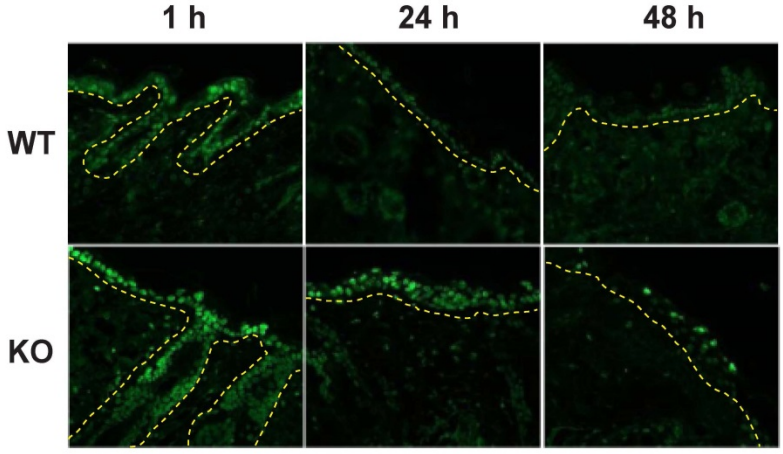


Figure 4. CPD levels in the skin of VDRKO and controls. Cyclobutane pyrimidine dimers (CPD) were determined by immunohistochemistry. Both wildtype and VDRKO littermates formed CPDs immediately after acute UVB exposure. However the VDRKO mice were slow to clear the CPDs which persisted at least for 48h, unlike the near total clearance by 24h in the wildtype mice.

Task 2. Determine whether mice lacking VDR but with constitutively active hedgehog (Hh) signaling (ptch null) show accelerated tumor formation following UVB treatment, whereas those with an inactivated Shh are protected from tumor formation. In this task we bred mice homozygous for the floxed VDR with mice homozygous for floxed Shh (which will inhibit Hh signaling when deleted). We did not study mice lacking ptch. Deletion of the VDR and Shh was accomplished by breeding the double floxed mice with ER^{T2}-K14-cre recombinase, and subsequently treating the mice at 4wks of age with tamoxifen that activates the cre recombinase as described in task 1. The breeding produces littermates which have or lack the ER^{T2}-K14-cre recombinase, and the latter serve as the controls. The survival of the ShhKO was poor, and only the acute response to UVB could be studied.

2a. *Develop mice with floxed ptch, floxed Shh, and the combination of each of these floxed genes with floxed VDR and breed them with mice expressing the appropriate floxed genes plus ER^{T2}-K14-cre recombinase.* As noted above, we developed mice only with floxed Shh and the combination of floxed VDR and Shh.
2b. *Treat the litters from the above breeding and those of the single floxed gene controls with 0.2mg tamoxifen/mouse intraperitoneally every other day for 3 injections beginning at 4wks of age.* This was done. The phenotype of these mice is shown in figure 5. These mice also lose hair, and develop loss of hair follicles. The skin is hyperproliferative. The ears become quite abnormal.

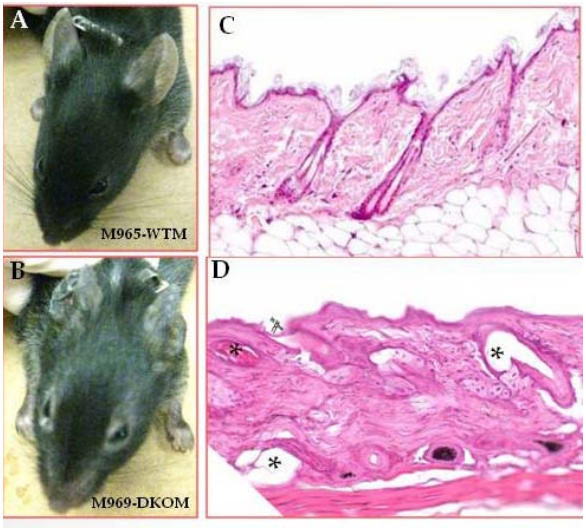


Figure 5. Appearance and histology of mice lacking both VDR and SHH in their epidermis. 8 wk old epidermal specific *Vdr* and *Shh* double-null mice demonstrate hair loss (prominent on ears and around eyes) and ragged ears 3 weeks after tamoxifen injection (B), versus normal hair/skin in control (A). H&E staining of the skin shows abnormal degenerating hair follicles with hyper-proliferation of sebaceous glands and epidermis (D) vs. control (C). Bar: 20x

2c. *Perform long treatment (40wks) of the mice with UVB starting at 5wks (1 week after tamoxifen injections).* We have not been able to treat these mice for the full 40wks because of a high mortality rate.

2d. *Perform acute treatment with a single dose of UVB starting at 5wks (1 week after tamoxifen injections).* These experiments have been performed, but the histologic examination of the skin samples has not been completed.

2e. *Complete data analysis and reporting*. We are in the process of finishing the data analysis and writing up the results for the DKO mice for which we have the full set of both acute and chronic UVB exposure. We are in the process of analyzing the acute UVB exposure data for essentially all other genotypes, and expect to have those data completed within the next several months, but beyond the duration of this funding cycle.

Key Research Accomplishments

1. Deletion of β-catenin or Shh even after the developmental hair follicle cycle still has a pronounced negative impact on hair follicle cycling as well as epidermal differentiation resulting in high rates of mortality.
2. Activation of β-catenin even after the developmental hair follicle cycle continues to result in increased but abnormal hair follicles with eventual loss of hair follicle cycling. Differentiation of the epidermis is likewise impacted resulting in reduced survival.
3. Surprisingly when mice lack β-catenin or Shh in combination with VDRKO their survival was increased. This result remains under investigation.
4. Deletion of β-catenin does not appear to protect the skin from UVB induced tumors in mice also lacking the VDR, contrary to expectations.
5. Loss of VDR in the keratinocyte results in a hyperproliferative response to UVB with decreased clearance of CPDs

Reportable Outcomes to date

Manuscripts and presentations:

1. Bikle DD 2012. Protective actions of vitamin D in UVB induced skin cancer. Photochem Photobiol Sci 11: 1808 – 1816
2. Bikle DD and Jiang Y. 2013. The protective role of vitamin D signaling in non-melanoma skin cancer. Cancers 5:1426-1438.

3. Jiang YJ, Teichert AE, Fong C, Oda Y, Bikle DD. 2013 1,25(OH)2-Dihydroxyvitamin D3/VDR protects the skin from UVB-induced tumor formation by interacting with the β-catenin pathway. J Ster Biochem Molec Biol 136: 229-232
4. Bikle DD, Elalieh H, Welsh J, Oh D, Cleaver J, Teichert A. 2013 Protective role of vitamin D signaling in skin cancer formation. J Ster Biochem Molec Biol 136: 271-279.
5. Bikle DD 2013. The vitamin D receptor: a tumor suppressor in skin In: Sunlight, Vitamin D and Skin Cancer, 2nd Edition ed by Reichrath J, Landes Bioscience (in press)
6. Jiang YJ, Bikle DD 2013. Long non-coding RNA: a novel mechanism for the protective role of vitamin D signaling in skin cancer formation. Oral presentation at the 16th Vitamin D Workshop, June 2013.
7. Jiang YJ, Bikle DD 2013. Long non-coding RNA: a new player in VDR protection against skin cancer formation. Experimental Dermatology 23:147-150
8. Jiang YJ, Bikle DD 2014. Long non-coding RNA profiling reveals new mechanism of VDR protection skin cancer formation. Journal of Steroid Biochemistry and Molecular Biology (in press).

Development of novel bioengineered mice

1. Conditional epidermal specific VDR null mouse
2. Conditional epidermal specific β–catenin null mouse
3. Conditional epidermal specific VDR/ β-catenin null mouse
4. Conditional epidermal specific constitutively activated (ca) β-catenin mouse
5. Conditional epidermal specific VDR null/ca β-catenin mouse
6. Conditional epidermal specific Shh null mouse
7. Conditional epidermal specific VDR/Shh null mouse

Funding applied for: NIH RO1: Disruption of hedgehog and beta-catenin signaling in VDR/CASR null epidermis

Conclusions

We have developed a number of bioengineered strains of mice that serve as models for over expression or under expression of the HH and wnt/β-catenin pathways, to determine whether these pathways would alter the susceptibility of the VDR null mouse to UVB induced epidermal cancer. Much of the first year was devoted to developing these mouse models, which is now achieved, whereas the second year has been devoted to performing the UVB exposure studies. We were limited in the chronic UVB studies by the poor survival of a number of the genotypes despite the fact that the gene deletions were performed post weaning. That in itself is new and reportable data, and our histologic and functional examination of the hair follicle and epidermis in these mice is ongoing. In the case of the epidermal specific VDR/ β-catenin null mouse, we did not find the protection we anticipated in that these mice did develop tumors over the 40wk period of UVB exposure. We have also had to modify our breeding strategy in some models in that homozygous expression of the active form of β-catenin proved excessive leading to decreased survival. However, we have performed short term UVB exposures for most of the models, and are analyzing the results, but these analyses have not been completed by the time this funding cycle has ended.

References

1. Zinser GM, Sundberg JP, Welsh J. Vitamin D3 receptor ablation sensitizes skin to chemically induced tumorigenesis. Carcinogenesis 23:2103-2109, 2002
2. Bikle DD, Elalieh H, Chang S, Xie Z, Sundberg J 2006 Development and progression of alopecia in the vitamin D receptor null mouse. J Cell Physiol 207:340-353
3. Teichert A, Elalieh H, Elias P, Welsh J, Bikle DD. 2011. Over-expression of hedgehog signaling is associated with epidermal tumor formation in *vitamin D receptor* null mice. J Invest Dermatol 131:2289-2297

4. Bikle DD, Oda Y, and Arnaud Teichert A 2011The Vitamin D Receptor: a Tumor Suppressor in Skin. Discovery Medicine 11:7-17
5. Bikle DD 2012. Protective actions of vitamin D in UVB induced skin cancer. Photochem Photobiol Sci 11: 1808 – 1816
6. Bikle DD and Jiang Y. 2013. The protective role of vitamin D signaling in non-melanoma skin cancer. Cancers 5:1426-1438.
7. Jiang YJ, Teichert AE, Fong C, Oda Y, Bikle DD. 2013 1,25(OH)2-Dihydroxyvitamin D3/VDR protects the skin from UVB-induced tumor formation by interacting with the β-catenin pathway. J Ster Biochem Molec Biol 136: 229-232
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